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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68	A2	(11) International Publication Number: WO 96/10648 (43) International Publication Date: 11 April 1996 (11.04.96)
(21) International Application Number: PCT/US95/12608 (22) International Filing Date: 28 September 1995 (28.09.95) (30) Priority Data: 08/316,544 30 September 1994 (30.09.94) US (71) Applicant: PROMEGA CORPORATION [US/US]; 2800 Woods Hollow Road, Madison, WI 53711 (US). (72) Inventors: SCHUMM, James, W.; 5943 Timber Ridge Trail, Madison, WI 53711 (US). SPRECHER, Cynthia, J.; 2121 South Whitney Way, Madison, WI 53711 (US). LINS, Ann, M.; 438 Lueders Road, Sauk City, WI 53583 (US). (74) Agent: SARA, Charles, S.; Dewitt Ross & Stevens, S.C., 8000 Excelsior Drive, Madison, WI 53717-1914 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI**(57) Abstract**

The present invention is directed to the simultaneous amplification of multiple distinct genetic loci using PCR or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex.

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MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

FIELD OF THE INVENTION

The present invention is generally directed to the detection of genetic markers in a genomic system. The present invention is more specifically directed to the simultaneous amplification of multiple distinct polymorphic genetic loci using the polymerase chain reaction or other
5 amplification systems to determine in one reaction the alleles of each locus contained within the multiplex system.

CITED REFERENCES

A full bibliographic citation of the references cited in this application can be found in the section preceding the claims.

DESCRIPTION OF THE PRIOR ART

In recent years, the discovery and development of polymorphic short tandem repeats (STRs) as genetic markers has stimulated progress in the development of linkage maps, the identification and characterization of diseased genes,
15 and the simplification and precision of DNA typing.

Many loci, at least in the human genome, contain a polymorphic STR region. STR loci consist of short, repetitive sequence elements of 3 to 7 base pairs in length. It is estimated that there are 2,000,000 expected
20 trimeric and tetrameric STRs present as frequently as once every 15 kilobases (kb) in the human genome (Edwards et al. 1991; Beckmann and Weber 1992). Nearly half of the STR loci studied by Edwards et al. (1991) are polymorphic, which provides a rich source of genetic markers. Variation
25 in the number of repeat units at a particular locus is responsible for the observed polymorphism reminiscent of VNTR loci (Nakamura et al. 1987) and minisatellite loci (Jeffreys et al. 1985), which contain longer repeat units, and microsatellite or dinucleotide repeat loci (Litt and
30 Luty 1989, Tautz 1989, Weber and May 1989, Beckmann and Weber 1992).

amplified material. Examples of this include radioactive or fluorescent labeling of one of the two primers prior to the amplification of a locus. One of the more sophisticated approaches to detection is the use of
5 different fluorescent labels to allow detection of amplified materials representing different loci, but existing in the same space following electrophoresis. The products of the different loci are differentiated with the use of filters, which allow visualization of one
10 fluorescent label at a time.

Reference is made to International Publications WO 93/18177 and WO 93/18178 to Fortina et al., which are directed to methods and kits for diagnosing diseases such as Cystic Fibrosis and β -thalassemia, respectively, using
15 an allele-specific multiplex polymerase chain reaction system. According to Fortina et al., multiplex PCR has also been used for simultaneous amplification of multiple target sequences, permitting mutant allele scanning using two lanes of an agarose gel.

20 Ballabio et al. (1991), disclose a single-tube, multiplex allele-specific PCR test using two different dye-tagged fluorescent primers for detection of the Δ F508 cystic fibrosis mutation.

While there are multiplex amplification procedures for
25 specific loci, the use of multiplex amplification procedures is greatly desired for the detection of alleles in other types of loci such as specific STR loci.

SUMMARY OF THE INVENTION

30 It is therefore an object of the present invention to provide a method for the simultaneous amplification of multiple distinct polymorphic STR loci using PCR or other amplification systems to determine, in one reaction, the alleles of each locus contained within the multiplex.
35 These combinations of specific loci into multiplexes have not been heretofore shown.

HUMCD4; HUMCSF1PO, HUMTH01 and HUMVWFA31; HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31; HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX, HUMTH01 and HUMCD4; and
5 HUMCSF1PO, HUMTH01, HUMTPOX and HUMCD4.

The present invention provides a high throughput method for the detection and analysis of polymorphic genetic markers using specific combinations of loci and specified conditions. By selection of the appropriate
10 detection method, the process can be used in laboratories which have only a power supply and a standard apparatus for polyacrylamide gel electrophoresis or those which have the latest in equipment for fluorescent gel scanning, e.g., FluorImager™-575 (Molecular Dynamics, Sunnyvale, CA). Thus,
15 the process of the present invention is adaptable for a variety of uses and laboratories.

The approach as specified in the present invention produces a savings in time, labor and materials in the analysis of loci contained within the multiplexes. The
20 process of the present invention includes all the requisite primers, allowing between two and four or more loci to be amplified together in one amplification tube instead of amplifying each locus independently.

The present invention has specific use in the field of forensic analysis, paternity determination, monitoring of
25 bone marrow transplantation, linkage mapping, and detection of genetic diseases and cancers.

These and other aspects of the present invention will become evident upon reference to the following detailed
30 description of the invention and the attached drawings and photographs.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph illustrating the simultaneous
35 amplification of three loci: HUMCSF1PO, HUMTPOX and HUMTH01, with the amplified products of each locus shown

Figure 18 is a photograph showing the silver stain detection of the multiplex amplification in Example 18.

Figure 19 is a photograph showing the silver stain detection of the multiplex amplification in Example 19.

5 Figure 20 is a photograph showing the silver stain detection of the multiplex amplification in Example 20.

Figure 21 is a photograph showing the silver stain detection of the multiplex amplification in Example 21.

10 Figure 22 is a photograph showing the silver stain detection of the multiplex amplification in Example 22.

Figure 23 is a photograph showing the silver stain detection of the multiplex amplification in Example 23.

Figure 25 is a photograph showing the silver stain detection of the multiplex amplification in Example 25.

15 Figure 26 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 26.

Figure 27 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in
20 Example 27.

Figure 28 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 28.

25 Figure 29 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 29.

Figure 30 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 30.

30 Figure 31 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 31.

Figure 32 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in
35 Example 32.

measure generally displays smaller values than the other commonly used measure, i.e., heterozygosity. For markers that are highly informative (heterozygosities exceeding about 70%), the difference between heterozygosity and PIC is slight.

Primary reaction: initial reaction using the purified human genomic DNA as template for the PCR.

Primers: two single-stranded oligonucleotides or DNA fragments which hybridize with opposing strands of a locus such that the 3' termini of the primers are in closest proximity.

Primer pair: two primers including primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

Primer site: the area of the target DNA to which a primer hybridizes.

Secondary reaction: reamplification with the same or different primer pair using a dilution of the primary reaction as template for the PCR.

Construction of the Multiplex System

Prior to constructing the multiplex system, an appropriate set of loci, primers, and amplification protocols must be selected such that amplification generates fragments such that alleles of the various loci do not overlap in size or, when such overlap occurs, fragments representing different loci are detectable by separate means. In addition, the selected loci must be compatible for use with a single amplification protocol. The specific combinations of loci described herein are unique in this application. Combinations of loci may be rejected for either of these reasons, or because, in combination, one or more of the loci do not produce adequate product yield, or fragments which do not represent authentic alleles are produced in this reaction.

25. HUMCSF1PO, HUMTPOX and HUMCD4;
26. HUMHPRTB, HUMFESFPS and HUMMYOPK (Myotonic);
27. HUMCSF1PO, HUMTH01 and HUMCD4;
28. HUMCSF1PO, HUMTH01 and HUMVWFA31;
- 5 29. HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL;
30. HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31;
31. HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and HUMLIPOL;
32. HUMCSF1PO, HUMTPOX, HUMTH01 and HUMCD4; and
- 10 33. HUMCSF1PO, HUMTH01, HUMTPOX and HUMCD4.

The primers must also be designed so that the size of the resulting amplification products differ in length, thereby facilitating assignment of alleles to individual loci during detection. Inappropriate selection of primers can produce several undesirable effects such as lack of amplification, amplification at multiple sites, primer dimer formation, undesirable interaction of primer sequences from different loci, production of alleles from one locus which overlap with alleles from another, or the need for amplification conditions or protocols for the different loci which are incompatible in a multiplex. The synthesis of the primers is conducted by procedures known to those skilled in the art.

Using Multiplexes of Two Loci to Develop Multiplexes Using More than Two Loci

Once a multiplex containing two loci is developed, it may be used as a core to create multiplexes containing more than two loci. New combinations are created including the first two loci. For example, the core multiplex containing loci HUMTH01 and HUMCSF1PO was used to generate derivative multiplexes of HUMTH01, HUMCSF1PO, and HUMTPOX; HUMTH01, HUMCSF1PO, and HUMCD4; HUMTH01, HUMCSF1PO, and HUMVWFA31; HUMTH01, HUMCSF1PO, HUMVWFA31, and HUMTPOX; and HUMTH01, HUMCSF1PO, HUMCD4, and HUMTPOX. Many other derivative multiplexes can be generated based upon a working

	Designation	Primer sequences	Sequence ID Number
	HSAC04 (ACTBP2)	primer 1: ACA TCT CCC CTA CCG CTA TA primer 2: AAT CTG GGC GAC AAG AGT GA	1 2
5	HUMAPOA2 (APOCIII)	primer 1: GGA GCA GTC CTA GGG CCG CGC CGT primer 2: GTG ACA GAG GGA GAC TCC ATT AAA	3 4
	HUMCSF1PO	primer 1: AAC CTG AGT CTG CCA AGG ACT AGC primer 2: TTC CAC ACA CCA CTG GCC ATC TTC	5 6
	HUMCYP19 (CYARP450)	primer 1: GCA GGT ACT TAG TTA GCT AC primer 2: TTA CAG TGA GCC AAG GTC GT	7 8
10	HUMCD4	primer 1: CCA GGA AGT TGA GGC TGC AGT GAA primer 2: TTG GAG TCG CAA GCT GAA CTA GCG	9 10
	HUMF13A01	primer 1: GAG GTT GCA CTC CAG CCT TTG CAA primer 2: TTC CTG AAT CAT CCC AGA GCC ACA	11 12
	HUMBFXIII (F13B)	primer 1: TGA GGT GGT GTA CTA CCA TA primer 2: GAT CAT GCC ATT GCA CTC TA	13 14
	HUMFABP	primer 1: GTA GTA TCA GTT TCA TAG GGT CAC C primer 2: CAG TTC GTT TCC ATT GTC TGT CCG	15 16
15	HUMFESFPS	primer 1: GCT GTT AAT TCA TGT AGG GAA GGC primer 2: GTA GTC CCA GCT ACT TGG CTA CTC	17 18
	HUMHPRTB (HPRT-1)	primer 1: ATG CCA CAG ATA ATA CAC ATC CCC primer 2: CTC TCC AGA ATA GTT AGA TGT AGG	19 20
	HUMMYOPK Myotonic	primer 1: GCT CGA AGG GTC CTT GTA GCC GGG primer 2: GAT AGG TGG GGG TGC GTG GAG GAT	21 22
20	HUMLIPOL	primer 1: CTG ACC AAG GAT AGT GGG ATA TAG primer 2: GGT AAC TGA GCG AGA CTG TGT CT	23 24
	HUMPLA2A1 (PLA-AZ)	primer 1: GGT TGT AAG CTC CAT GAG GTT AGA primer 2: TTG AGC ACT TAC TAT GTG CCA GGC T	25 26
	HUMTH01	primer 1: GTG GGC TGA AAA GCT CCC GAT TAT primer 2: ATT CAA AGG GTA TCT GGG CTC TGG	27 28
25	HUMTPOX	primer 1: ACT GGC ACA GAA CAG GCA CTT AGG primer 2: GGA GGA ACT GGG AAC CAC ACA GGT	29 30
	HUMVWFA31	primer 1: GA AAG CCC TAG TGG ATG ATA AGA ATA ATC primer 2: GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG	31 32

Reference is made to the examples below for additional details of the specific procedure relating to each multiplex. The locus-specific primers include a number of nucleotides which, under the conditions used in the hybridization, are sufficient to hybridize with an allele of the locus to be amplified and to be essentially free from amplification of alleles of other loci. Reference is made to U. S. Patent 5,192,659 to Simons, which is incorporated herein by reference for a more detailed description of locus-specific primers.

Separation and Detection of DNA Fragments

Following amplification, products are then separated by electrophoresis, e.g., denaturing polyacrylamide gel electrophoresis (Sambrook et al., 1989). Preferred gel preparation and electrophoresis procedures are conducted as

products can be achieved. For fluorescent analyses, one fluoresceinated primer can be included in the amplification of each locus. Separation of the amplified fragments is achieved in precisely the same manner as with the silver stain detection method. The resulting gel is loaded onto a FluorImager® 575 (Molecular Dynamics, Sunnyvale, CA) which scans the gel and digitizes the data in three minutes. The FluorImager® contains an argon laser emitting 488 nm light which sweeps through the gel using a galvanometer-controlled mirror. The light activates fluorescent molecules in its path and they, in turn, emit light of higher wavelength. A filter prohibits passage of the original light, but allows collection of the emitted light by a fiber optic collector. A second filter selected by the user may be inserted between the fiber optic collector and the photomultiplier, allowing detection of specific wavelength bands (or colors) with each scan.

The image has an overall cleaner appearance than that obtained with the silver stain for three reasons. First, only one of the two PCR product strands is labeled with primer, simplifying the two band per allele images of the silver stain. Second, in the silver stain reaction, the entire gel is exposed to silver and prone to silver deposition causing a significant general background. With the fluorescent reporter, only the primer is labeled and the unincorporated primers migrate out of the bottom of the gel prior to detection. Third, some artifact bands of the PCR reaction are plentiful, but contain very little primer.

Because this fluorescent method detects only products with one particular primer, some of these artifacts which appear in silver stain of multiplex amplifications are not detected. In fact, this characteristic has allowed development of the more complex quadriplex as shown in Figure 2 in place of the triplex shown in Figure 1.

polyacrylamide gel (19:1 ratio of acrylamide to bis-acrylamide) which contained 7 M urea (Sambrook et al., 1989) and was chemically cross-linked to one glass plate (Kobayashi, 1988). DNA samples were mixed with 3 μ l loading solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 2 min., and chilled on ice prior to loading.

Electrophoresis was performed at 60 W in 0.5x TBE for 1-2 hrs. The DNA was detected by silver staining (Bassam et al., 1991). Permanent images were obtained by exposure to Electrophoresis Duplicating Films (EDF, Kodak, Cat.No. 809 6232). Alternatively, detection can be performed by fluorescent scanning (Schumm et al., 1994) or radioactive detection (Hammond et al., 1994).

EXAMPLE 1

Silver Stain Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, and HUMTH01

In this example, a DNA template (three DNA samples) was amplified at the individual loci HUMCSF1PO, HUMTPOX, and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were performed in 50 μ l volumes using 25ng template, 0.03U Taq DNA Polymerase/ μ l, 1x STR Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP), and using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1 (96°C for 2 min., then 10 cycles of 94°C for 1 min., 64°C for 1 min., and 70°C for 1.5 min., followed by 20 cycles of 90°C for 1 min., 64°C for 1 min., 70°C for 1.5 min.) was employed.

Six amplification primers were used in combination, including 0.2 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 0.2 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 0.6 μ M each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28].

Amplified products were separated by denaturing acrylamide gel electrophoresis on a 40cm gel for 60-90 min.

HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31. Lane 1 contains allelic ladders for the 4 loci.

EXAMPLE 3

5

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMVWFA31

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed in 25 μ l volumes using 25ng template, 0.03U Taq DNA Polymerase/ μ l, 1x STR Buffer (described in example 1), and a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2 (96°C for 2 min., then 10 cycles of 94°C for 1 min., 60°C for 1 min., and 70°C for 1.5 min., followed by 20 cycles of 90°C for 1 min., 64°C for 1 min., 70°C for 1.5 min.) was employed. Amplified products were separated by denaturing acrylamide gel electrophoresis on a 32cm gel for 45 min. at 40 W and products were visualized by silver stain analysis according the protocol of Bassam et al. (supra.). Six primers were used in combination including 0.2 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 1.5 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 1 μ M each HUMVWFA31 primers 1 [SEQ. ID. 31] and 2 [SEQ. ID. 32].

Reference is made to Figure 3 which reveals the silver stain detection of the multiplex amplification. Lanes 2-6 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMVWFA31. Lanes 1 and 7 contain allelic ladders for the 3 loci.

EXAMPLE 4

Fluorescent Detection of Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL

35

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as

sample without DNA subjected to the same procedures, i.e., a negative control.

EXAMPLE 6

5 Multiplex Amplification of Loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1

 In this example, a DNA template was amplified at the
 loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1
10 simultaneously in a single reaction vessel. The PCR
amplifications were performed in 15 μ l volumes with 25ng
template, 0.02U Taq DNA Polymerase/ μ l, 1x Taq DNA
Polymerase Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at
25°C), 0.1% Triton X-100 and 1.5mM MgCl₂) and 200 μ M each of
15 dATP, dCTP, dGTP and dTTP using a Thermal Cycler 480
(Perkin Elmer Cetus). Amplification protocol 2, as
described in Example 3, was employed. Amplified products
were separated and detected per example 1. Six primers
were used in combination, including 1 μ M each HSAC04
20 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], 1 μ M
each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26],
and 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ.
ID. 8].

 Reference is made to Figure 6 which reveals the silver
25 stain detection of the multiplex amplification. Lanes 1-3
contain DNA samples simultaneously co-amplified for the
loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1. Lane 4
displays a sample without DNA subjected to the same
procedures, i.e., a negative control.

30

EXAMPLE 7

Multiplex Amplification of Loci HSAC04 (ACTBP2) and HUMFABP

35 In this example, a DNA template was amplified at the
loci HSAC04 (ACTBP2) and HUMFABP simultaneously in a single
reaction vessel. The PCR amplifications and other
manipulations were performed as described in Example 5
using amplification protocol 2, as described in Example 3.

single reaction vessel. The PCR amplifications were performed in 50 μ l volumes with 25ng template, 0.02U Taq DNA Polymerase/ μ l, 1x Taq DNA Polymerase Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100 and 1.5mM MgCl₂) and 200 μ M each of dATP, dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1, as described in Example 1, was employed. Amplified products were separated and detected as described in Example 1. Six primers were used in combination, including 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10], 1 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], and 1 μ M each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28].

Reference is made to Figure 9 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCD4, HUMCSF1PO, and HUMTH01. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

EXAMPLE 10

Multiplex Amplification of Loci HUMCYP19, HUMFABP, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HUMCYP19, HUMFABP, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], 1 μ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16] and 1 μ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

Reference is made to Figure 10 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCYP19, HUMFABP, and HUMPLA2A1. Lane 4

for the loci HUMF13A01 and HUMFABP. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

EXAMPLE 13

5

Multiplex Amplification of Loci HUMBFXIII (F13B) and HUMFESFPS

In this example, a DNA template was amplified at the loci HUMBFXIII (F13B) and HUMFESFPS simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 1, as described in Example 1. Four primers were used in combination, including 1 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14], and 1 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18].

Reference is made to Figure 13 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B) and HUMFESFPS. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

EXAMPLE 14

25

Multiplex Amplification of Loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14], 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], and 1 μ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

Reference is made to Figure 14 which reveals the silver stain detection of the multiplex amplification.

1.5 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18].

Reference is made to Figure 16 which reveals the silver stain detection of the multiplex amplification. Lanes 1-6 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB and HUMFESFPS using 500, 50, 25, 5, 1 and 0.5ng DNA template. Lane 7 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

10

EXAMPLE 17

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMLIPOL

15 In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. 20 Six primers were used in combination, including 0.4 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 3 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 2 μ M each HUMLIPOL primers 1 [SEQ. ID. 23] and 2 [SEQ. ID. 24].

25 Reference is made to Figure 17 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS and HUMLIPOL. Lane 4 displays a sample without DNA subjected to the same 30 procedures, i.e., a negative control.

EXAMPLE 18

Multiplex Amplification of Loci HUMBFXIII (F13B) and HUMLIPOL

35

In this example, a DNA template was amplified at the loci HUMBFXIII (F13B) and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1

combination, including 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 2 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 2 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

5 Reference is made to Figure 20 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B).

10 EXAMPLE 21

Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, and HUMCD4

15 In this example, a DNA template was amplified at the loci HUMCSF1PO, HUMTPOX, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 1, as described in Example 1. Six primers were used in combination, including 1 μ M each
20 HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 1 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

25 Reference is made to Figure 21 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, and HUMCD4.

30 EXAMPLE 22

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMMYOPK (Myotonic)

35 In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMMYOPK simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 1 μ M

1 [SEQ. ID. 11] and 2 [SEQ. ID. 12] and 1 μ M each HUMMYOPK (Myotonic) primers 1 [SEQ. ID. 21] and 2 [SEQ. ID. 22].

Reference is made to Figure 24 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMMYOPK (Myotonic).

EXAMPLE 25

Multiplex Amplification of Loci HUMF13A01 and HUMBFXIII (F13B)

In this example, a DNA template was amplified at the loci HUMF13A01 and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 0.03U Taq DNA Polymerase/ μ l and amplification protocol 2, as described in Example 3. Four primers were used in combination, including 0.1 μ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12] and 0.5 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

Reference is made to Figure 25 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMBFXIII (F13B).

EXAMPLE 26

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4

In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.04U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in Example 1. Eight amplification primers were used in combination, including 2 μ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 0.5 μ M each HUMTPOX primer 1 [SEQ.

simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in Example 1. Six amplification primers were used in combination, including 1 μ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27], and 1 μ M each HUMVWFA31 primer 1 [SEQ. ID. 31] and fluorescein-labeled primer 2 [SEQ. ID. 32].

Amplified products were detected as in Example 2. Reference is made to Figure 28 which is a photograph of a computer image of a FluorImager scan. Lanes 1 and 2 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTH01, and HUMVWFA31.

EXAMPLE 29

Fluorescent Detection of Multiplex Amplification of Loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL

In this example, a DNA template was amplified at the individual loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.03U Taq DNA Polymerase/ μ l and amplification protocol 2, as described in Example 3. Six amplification primers were used in combination, including 1 μ M each HUMHPRTB primer 2 [SEQ. ID. 20] and fluorescein-labeled primer 1 [SEQ. ID. 19], 1 μ M each HUMBFXIII (F13B) primer 2 [SEQ. ID. 14] and fluorescein-labeled primer 1 [SEQ. ID. 13], and 1 μ M each HUMLIPOL primer 2 [SEQ. ID. 24] and fluorescein-labeled primer 1 [SEQ. ID. 23].

Amplified products were detected as in Example 2. Reference is made to Figure 29 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL.

EXAMPLE 32

Fluorescent Detection of Multiplex Amplification
of Loci HUMTH01 and HUMTPOX

- 5 In this example, a DNA template was amplified at the individual loci HUMTH01 and HUMTPOX simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in
- 10 Example 1. Four amplification primers were used in combination, including 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 μ M each HUMTPOX primer 2 [SEQ. ID. 30] and fluorescein-labeled primer 1 [SEQ. ID. 29].
- 15 Amplified products were detected as in Example 2. Reference is made to Figure 32 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMTH01 and HUMTPOX.
- 20 It is understood that the invention is not confined to the particular construction and arrangements herein illustrated and described, but embraces such modified forms thereof and come within the scope of the claims following the bibliography.

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Weber, J. L. and May, P. E. (1989) "Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction," *Am. J. Hum. Genet.* 44: 388-396.

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATCTGGGCG ACAAGAGTGA

20

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAGCAGTCC TAGGGCCGCG CCGT

24

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGACAGAGG GAGACTCCAT TAAA

24

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCAGGAAGTT GAGGCTGCAG TGAA

24

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTGGAGTCGC AAGCTGAACT AGCG

24

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGGTTGCAC TCCAGCCTTT GCAA

24

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTCCTGAATC ATCCCAGAGC CACA

24

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTGTTAATT CATGTAGGGA AGGC

24

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTAGTCCCAG CTACTTGGCT ACTC

24

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATGCCACAGA TAATACACAT CCCC

24

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTCTCCAGAA TAGTTAGATG TAGG

24

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

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24

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

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25

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTGGGCTGAA AAGCTCCCGA TTAT

24

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ATTCAAAGGG TATCTGGGCT CTGG

24

CLAIMS

What is claimed is:

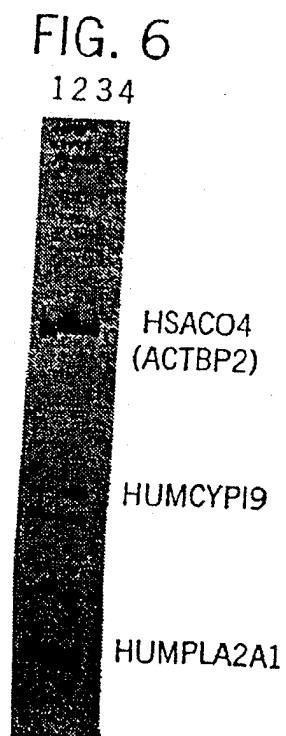
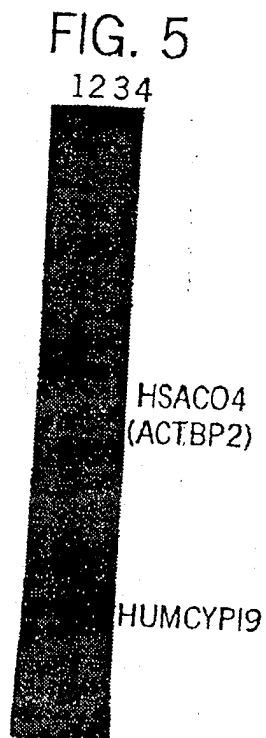
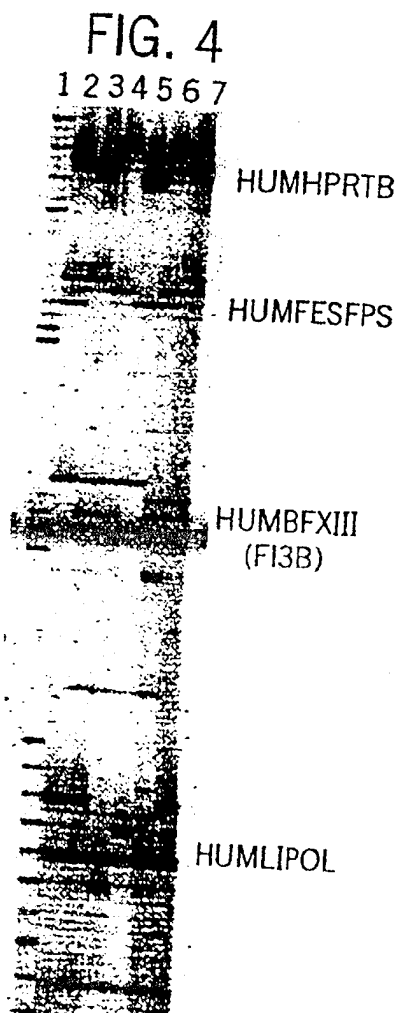
1. A method of simultaneously determining the alleles present in at least two loci from one or more DNA samples, comprising:
 - a. obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together;
 - b. amplifying the short tandem repeat sequences in the DNA sample; and
 - c. evaluating the amplified fragments to determine the alleles present at each amplified locus within the DNA sample.
2. The method of claim 1 wherein at least one of the loci is selected from the group consisting of: HUMCSF1PO, HUMTPOX, HUMVWFA31, HUMFESFPS, HUMBFXIII (F13B), HUMLIPOL, HSAC04 (ACTBP2), HUMCYP19, HUMAPOA2, HUMF13A01 and HUMMYOPK (Myotonic).
3. The method of claim 1 wherein at least two loci are selected from the groups consisting of: HUMTH01 and HUMCSF1PO; HUMTH01 and HUMCD4; HUMTH01 and HUMTPOX; HUMF13A01 and HUMFABP; HUMF13A01 and HUMMYOPK (Myotonic); HUMF13A01 and HUMBFXIII (F13B); HUMBFXIII (F13B) and HUMFESFPS; HUMBFXIII (F13B) and HUMLIPOL; HUMHPRTB and HUMFESFPS; HSAC04 (ACTBP2) and HUMCYP19; and HSAC04 (ACTBP2) and HUMFABP.
4. The method of claim 1 wherein the loci are selected from the group consisting of: HUMTH01 and HUMCSF1PO; HUMTH01 and HUMCD4; HUMTH01 and HUMTPOX; HUMF13A01 and HUMFABP; HUMF13A01 and HUMMYOPK (Myotonic); HUMF13A01 and HUMBFXIII (F13B); HUMBFXIII (F13B) and HUMFESFPS; HUMBFXIII (F13B) and HUMLIPOL; HUMHPRTB and HUMFESFPS; HSAC04 (ACTBP2) and HUMCYP19; HUMCSF1PO, HUMTPOX, and HUMTH01; HUMHPRTB, HUMFESFPS and HUMVWFA31; HSAC04

9. The method of claim 1 wherein the loci are HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and HUMLIPOL.
10. The method of claim 1 wherein the loci are HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31.
11. The method of claim 10, wherein in said amplifying step, amplification is primed with primer pairs comprising SEQ ID. NO. 5 and SEQ ID. NO. 6, SEQ ID. NO. 29 and SEQ ID. NO. 30, SEQ ID. NO. 27 and SEQ ID. NO. 28, and SEQ ID. NO. 31 and SEQ ID. NO. 32.
12. The method of claim 11, wherein the primers SEQ ID. NO. 5, SEQ ID. NO. 30, SEQ ID. NO. 27, and SEQ ID. NO. 32 are fluorescence labeled.
13. The method of claim 11, wherein the primer pairs SEQ ID. NO. 5 and SEQ ID. NO. 6 are present in a concentration of about $1\mu\text{M}$; primer pairs SEQ ID. NO. 29 and SEQ ID. NO. 30 are present in a concentration of about $0.15\mu\text{M}$, primer pairs SEQ ID. NO. 27 and SEQ ID. NO. 28 are present in a concentration of about $0.2\mu\text{M}$, and primer pair SEQ ID. NO. 31 and SEQ ID. NO. 32 are present in a concentration of about $1\mu\text{M}$.
14. The method of claim 1 wherein the loci are HUMHPRTB, HUMFESFPS and HUMVWFA31.
15. The method of claim 1 wherein the DNA in step b. is amplified by polymerase chain reduction.
16. The method of claim 10 wherein the process of amplifying short tandem repeat sequences requires primer pairs selected from the group consisting of SEQ ID. NO. 1 and SEQ ID. NO. 2, SEQ ID. NO. 3 and SEQ ID. NO. 4, SEQ ID. NO. 5 and SEQ ID. NO. 6, SEQ ID. NO. 7 and SEQ ID. NO. 8, SEQ ID. NO. 9 and SEQ ID. NO. 10,

tissue, placental cells or fetal cells present in amniotic fluid and mixtures of body fluids.

23. A method of simultaneously determining the alleles present in at least two loci from one or more DNA samples, comprising:
- 5 a. identifying an appropriate set of loci and primers which provide non-overlapping alleles;
 - b. obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together;
 - 10 c. amplifying the short tandem repeat sequences in the DNA sample; and
 - d. evaluating the amplified fragments to determine the alleles present at each amplified locus within the DNA sample.
24. A kit for simultaneously analyzing short tandem repeat sequences in at least two loci from one or more DNA samples, comprising:
- 5 a. a container containing oligonucleotide primer pairs for each of the specified loci; and
 - b. instructions for use.
25. The kit of claim 24 wherein the primer pairs are selected from the group of loci consisting of SEQ ID. NO. 1 and SEQ ID. NO. 2, SEQ ID. NO. 3 and SEQ ID. NO. 4, SEQ ID. NO. 5 and SEQ ID. NO. 6, SEQ ID. NO. 7 and
- 5 SEQ ID. NO. 8, SEQ ID. NO. 9 and SEQ ID. NO. 10, SEQ ID. NO. 11 and SEQ ID. NO. 12, SEQ ID. NO. 13 and SEQ ID. NO. 14, SEQ ID. NO. 15 and SEQ ID. NO. 16, SEQ ID. NO. 17 and SEQ ID. NO. 18, SEQ ID. NO. 19 and SEQ ID. NO. 20, SEQ ID. NO. 21 and SEQ ID. NO. 22, SEQ ID. NO. 23 and SEQ ID. NO. 24, SEQ ID. NO. 25 and SEQ ID. NO. 26, SEQ ID. NO. 27 and SEQ ID. NO. 28, SEQ ID. NO. 29 and SEQ ID. NO. 30, and SEQ ID. NO. 31 and SEQ ID. NO. 32.
- 10

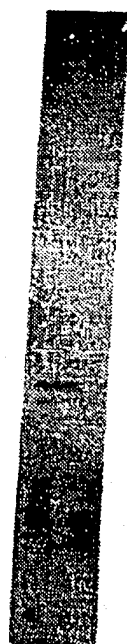
2 / 7



4/7

FIG. 13

1 2 3 4



HUMFESFPS

HUMBFXIII
(FI3B)

FIG. 14

1 2 3 4



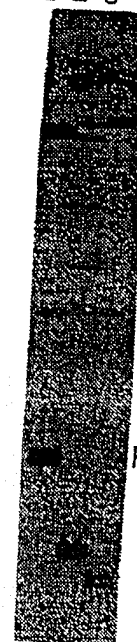
HUMHPRTB

HUMBFXIII
(FI3B)

HUMPLA2AI

FIG. 15

1 2 3 4



HUMFI3AOI

HUMFABP

HUMCD4

FIG. 16

1 2 3 4 5 6 7

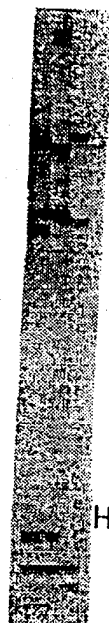


HUMHPRTB

HUMFESFPS

FIG. 17

1 2 3 4



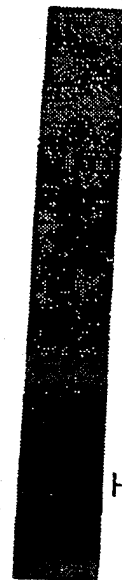
HUMHPRTB

HUMFESFPS

HUMLIPOL

FIG. 18

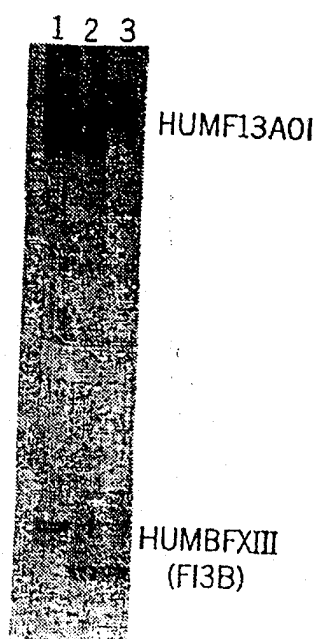
1 2 3



HUMBFXIII
(FI3B)

HUMLIPOL

FIG. 25



6/7
FIG. 26

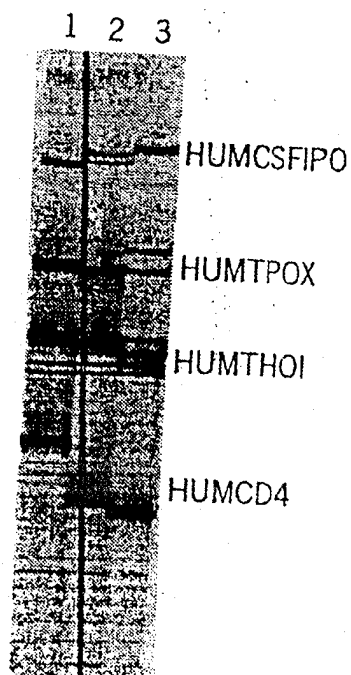


FIG. 27

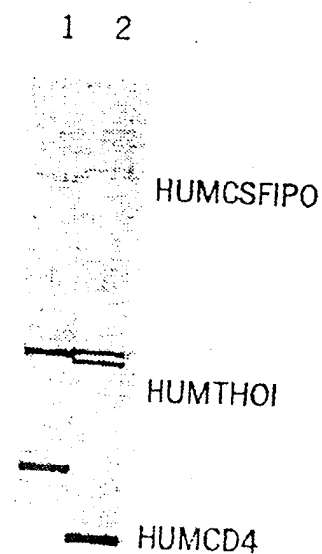


FIG. 28

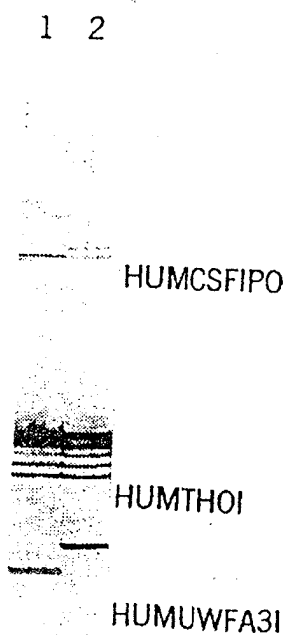


FIG. 29

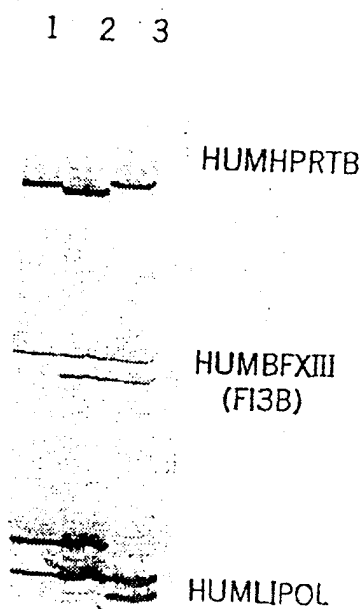
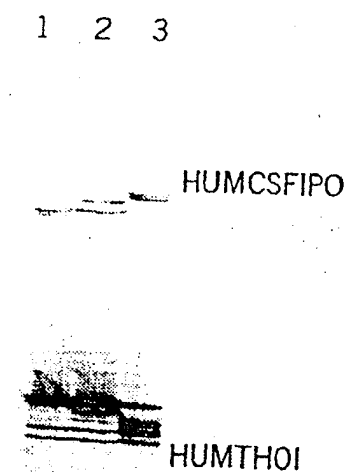


FIG. 30



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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12Q 1/68	A3	(11) International Publication Number: WO 96/10648 (43) International Publication Date: 11 April 1996 (11.04.96)
(21) International Application Number: PCT/US95/12608 (22) International Filing Date: 28 September 1995 (28.09.95) (30) Priority Data: 08/316,544 30 September 1994 (30.09.94) US (71) Applicant: PROMEGA CORPORATION [US/US]; 2800 Woods Hollow Road, Madison, WI 53711 (US). (72) Inventors: SCHUMM, James, W.; 5943 Timber Ridge Trail, Madison, WI 53711 (US). SPRECHER, Cynthia, J.; 2121 South Whitney Way, Madison, WI 53711 (US). LINS, Ann, M.; 438 Lueders Road, Sauk City, WI 53583 (US). (74) Agent: SARA, Charles, S.; Dewitt Ross & Stevens, S.C., 8000 Excelsior Drive, Madison, WI 53717-1914 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 27 February 1997 (27.02.97)
(54) Title: MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI		
(57) Abstract The present invention is directed to the simultaneous amplification of multiple distinct genetic loci using PCR or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex.		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/12608

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AMERICAN JOURNAL OF HUMAN GENETICS, vol. 55, no. 1, July 1994, CHICAGO, US, pages 175-189, XP000565532 HAMMOND ET AL.: "Evaluation of 13 STR loci for use in personal identification applications" cited in the application see the whole document	1-25
X	PCR METHODS & APPLICATIONS, vol. 3, 1993, ING HARBOR LABORATORY PRESS US, pages 13-22, XP000565542 KIMPTON ET AL.: "Automated DNA profiling employing multiplex amplification of STR loci" cited in the application see the whole document	1-25
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- "&" document member of the same patent family

Date of the actual completion of the international search

22 January 1997

Date of mailing of the international search report

27.01.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk

Authorized officer

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/12608

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUMAN MOLECULAR GENETICS, vol. 1, no. 2, 1992, page 137 XP002006999 ANKER ET AL.: "Tetranucleotide repeat polymorphism at the human thyroid peroxidase (hTPA) locus" see the whole document	2-4, 16, 25
Y	WO,A,92 21693 (US OF AMERICA AS PRESENTED BY) 10 December 1992 see figures 1,2,18,19,56	2-4, 16, 25
Y	WO,A,94 03640 (US HEALTH), 17 February 1994 Seq. Ids. 1, 2, 18 and 19	2-4, 16, 25
A	WO,A,92 13101 (INGENY B V) 6 August 1992 see page 6, line 16	2-4, 9, 16, 25

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 95/ 12608

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. Claims: 1, 6-8, 10-13, 15 and 17-24 (complete), 2-4, 16 and 25 (partly)

A method for simultaneously determining Short Tandem Repeat (STR) alleles from multiple loci in general and for groups of loci containing at least the HUMCSF1PO locus in particular as well as primers and kits used in this method.

2. Claims: 2-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups containing at least the HUMTPOX locus, excluding the groups of loci where also locus HUMCSF1PO is mentioned, as well as primers and kits used in this method.

3. Claims: 5, 9 and 14 (complete), 2-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the HUMBFXIII (F13B), HUMFESFPS and/or HUMF13A01 locus, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

4. Claims: 3-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the HUMTH01 and HUMCD4 loci, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/12608

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9213969	20-08-92	US-A- 5364759	15-11-94
		AU-B- 670231	11-07-96
		AU-A- 1334392	07-09-92
		CA-A- 2101859	01-08-92
		EP-A- 0639228	22-02-95
WO-A-9221693	10-12-92	US-A- 5378602	03-01-95
		AU-B- 663509	12-10-95
		AU-A- 2156992	08-01-93
		CA-A- 2110059	10-12-92
		EP-A- 0663923	26-07-95
		JP-T- 6509222	20-10-94
		US-A- 5468610	21-11-95
		US-A- 5369004	29-11-94
WO-A-9403640	17-02-94	US-A- 5369004	29-11-94
		AU-B- 673850	28-11-96
		AU-A- 4794693	03-03-94
		CA-A- 2141414	17-02-94
		EP-A- 0666928	16-08-95
WO-A-9213101	06-08-92	NL-A- 9100132	17-08-92
		EP-A- 0559841	15-09-93

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12Q 1/68	A3	(11) International Publication Number: WO 96/10648 (43) International Publication Date: 11 April 1996 (11.04.96)
(21) International Application Number: PCT/US95/12608 (22) International Filing Date: 28 September 1995 (28.09.95) (30) Priority Data: 08/316,544 30 September 1994 (30.09.94) US (71) Applicant: PROMEGA CORPORATION [US/US]; 2800 Woods Hollow Road, Madison, WI 53711 (US). (72) Inventors: SCHUMM, James, W.; 5943 Timber Ridge Trail, Madison, WI 53711 (US). SPRECHER, Cynthia, J.; 2121 South Whitney Way, Madison, WI 53711 (US). LINS, Ann, M.; 438 Lueders Road, Sauk City, WI 53583 (US). (74) Agent: SARA, Charles, S.; Dewitt Ross & Stevens, S.C., 8000 Excelsior Drive, Madison, WI 53717-1914 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i> <i>With amended claims.</i> (88) Date of publication of the international search report: 27 February 1997 (27.02.97) Date of publication of the amended claims: 27 March 1997 (27.03.97)
(54) Title: MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI		
(57) Abstract The present invention is directed to the simultaneous amplification of multiple distinct genetic loci using PCR or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex.		

AMENDED CLAIMS

[received by the International Bureau on 30 January 1997 (30.01.97);
original claims 2, 3, 6-8, 21 and 23 cancelled;
original claims 1, 4, 5, 9-11, 14-20, 22, 24 and 25 amended;
remaining claims unchanged (5 pages)]

1. A method of simultaneously determining the alleles present in at least two loci from one or more DNA samples, comprising:

5 a. obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together, and wherein the at least 2 loci are a pair of loci selected from the group of pairs consisting of:

HUMCSF1PO and HUMTH01;

10 HUMTPOX and HUMTH01;

HUMBFXIII (F13B) and HUMF13A01;

HUMBFXIII (F13B) and HUMFESFPS;

HUMBFXIII (F13B) and HUMLIPO;

HUMBFXIII (F13B) and HUMHPRTB;

15 HUMFESFPS and HUMHPRTB;

HUMF13A01 and HUMFABP;

HUMF13A01 and HUMMYOPK (Myotonic);

HSAC04 (ACTBP2) and HUMCYP19;

HSAC04 (ACTBP2) and HUMFABP;

20 HUMCYP19 and HUMPLA2A1; and

HUMTH01 and HUMCD4;

b. co-amplifying the at least two loci in the DNA sample in a multiplex amplification reaction; and

25 c. evaluating the co-amplified loci to determine the alleles present at each of the at least two loci within the DNA sample.

4. The method of claim 1 wherein the at least two loci are a triplex of three loci selected from the group of triplexes consisting of:

HUMCSF1PO, HUMTPOX, and HUMVWFA31;

5 HUMCSF1PO, HUMTPOX, and HUMCD4;

HUMCSF1PO, HUMTH01, and HUMCD4;

HUMCSF1PO, HUMTH01, and HUMVWFA31;

HUMTPOX, HUMTH01, and HUMCD4;

HUMTPOX, HUMTH01, and HUMVWFA31;

10 HUMTPOX, HUMHPRTB, and HUMBFXIII (F13B):

18. The method of claim 1 wherein the at least two co-amplified loci are evaluated by polyacrylamide gel electrophoresis.

19. The method of claim 18 wherein the at least two co-amplified loci are visualized by silver stain analysis.

20. The method of claim 18 wherein the at least two co-amplified loci are visualized by fluorescent analysis.

22. The method of claim 1 wherein the at least one DNA sample to be analyzed is selected from the group consisting of blood, semen, vaginal cells, hair, saliva, urine or other tissue, placental cells or fetal cells present in amniotic fluid and mixtures of body fluids.

24. A kit for simultaneously analyzing short tandem repeat sequences in at least two loci, comprising:

- a. a container containing oligonucleotide primer pairs for each of the at least 2 loci, wherein the at least two loci are a pair of loci selected from the group of pairs consisting of:

HUMCSF1B0 and HUMTH01;

HUMTPOX and HUMTH01;

HUMBFXIII (F13B) and HUMF13A01;

HUMBFXIII (F13B) and HUMFESFPS;

HUMBFXIII (F13B) and HUMLIPOL;

HUMBFXIII (F13B) and HUMHPRTB;

HUMFESFPS and HUMHPRTB;

HUMF13A01 and HUMFABP;

HUMF13A01 and HUMMYOPK (Myotonic);

HSAC04 (ACTBP2) and HUMCYP19;

HSAC04 (ACTBP2) and HUMFABP;

HUMCYP19 and HUMPLA2A1; and

HUMTH01 and HUMCD4;

- b. instructions for use.

15 HUMBFXIII (F13B), HUMLIPOL, and HUMHPRTB;
HUMBFXIII (F13B), HUMLIPOL, and HUMFESFPS;
HUMBFXIII (F13B), HUMHPRTB, and HUMFESFPS;
HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1;
HUMFESFPS, HUMHPRTB, and HUMVWFA31;
HUMFESFPS, HUMHPRTB, and HUMLIPOL;
HUMFESFPS, HUMHPRTB, and HUMMYOPK (Myotonic);
HUMF13A01, HUMFABP, and HUMCD4;
20 HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1;
HUMCYP19, HUMAPOA2, and HUMPLA2A1;
HUMCYP19, HUMPLA2A1, and HUMFABP; and
HUMCYP19, HUMPLA2A1, and HUMHPRTB.

5. The method of claim 1 wherein the loci are HUMFESFPS and HUMHPRTB.

9. The method of claim 1 wherein the at least two loci further includes a third and a fourth loci and wherein the at least two loci and the third and fourth loci are HUMBFXIII (F13B), HUMFESFPS, HUMLIPOL, and HUMHPRTB.

10. The method of claim 1 wherein the at least two loci further includes a third and a fourth loci and wherein the at least two loci and the third and fourth loci are HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31.

11. The method of claim 10, wherein in said co-amplifying step, amplification is primed with primer pairs comprising

5 SEQ ID. NO. 5 and SEQ ID. NO. 6,
SEQ ID. NO. 29 and SEQ ID. NO. 30,
SEQ ID. NO. 27 and SEQ ID. NO. 28, and
SEQ ID. NO. 31 and SEQ ID. NO. 32.

12. The method of claim 11, wherein the primers SEQ ID. NO. 5, SEQ ID. NO. 30, SEQ ID. NO. 27, and SEQ ID. NO. 32 are fluorescence labeled.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 95/ 12608

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

5. Claims: 2-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the HSAC04 (ACTBP2) and/or HUMCYP19 locus, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

6. Claims: 2-4, 16 and 25 (all partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the locus HUMVWFA31, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

7. Claims: 2-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the HUMLIPOI locus, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

8. Claims: 2-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the HUMAPOA2 locus, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

9. Claims: 2-4, 16 and 25 (partly)

A method for simultaneously determining STR alleles from multiple loci for groups of loci containing at least the HUMMYOPK (Myotonic) locus, excluding the groups where also loci of any of the preceding groups of claims are mentioned, as well as primers and kits used in this method.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/ 12608

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1,5-15,17-24 (complete) and 2-4,16,25 (partly)

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/12608

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL JOURNAL OF LEGAL MEDICINE, (9-1994) 107 (2) 77-89., XP000605266 LYGO ET AL.: "The validation of short tandem repeat (STR) loci for use in forensic casework." see the whole document ---	1-25
X	INTERNATIONAL JOURNAL OF LEGAL MEDICINE, (8-1994) 107 (1) 34-6., XP000605267 HOCHMEISTER ET AL.: "Swiss population data on three tetrameric short tandem repeat loci VWA, HUMTH01, and F13A1 derived using multiplex PCR and laser fluorescence detection." see the whole document ---	1-25
X	INTERNATIONAL JOURNAL OF LEGAL MEDICINE, (6-1994) 106 (6) 302-11., XP000604508 KIMPTON ET AL.: "Evaluation of an automated DNA profiling system employing multiplex amplification of four tetrameric STR loci." see the whole document ---	1-25
X	GENOMICS, vol. 13, 1992, SAN DIEGO, US, pages 375-380, XP000564673 HUANG ET AL.: "Genetic mapping of four dinucleotide repeat loci on the X chromosome using multiplex PCR" cited in the application see the whole document ---	1,15, 17-24
X	AMERICAN JOURNAL OF HUMAN GENETICS, vol. 49, 1991, CHICAGO, US, pages 951-960, XP000565533 CLEMENS ET AL.: "Carrier detection and prenatal diagnosis in D and BMD families, using dinucleotide repeat polymorphisms" cited in the application see the whole document ---	1,15, 17-24
X	GENOMICS, vol. 12, 1992, SAN DIEGO, US, pages 241-253, XP000565530 EDWARDS ET AL.: "Genetic variation at 5 trimeric and tetrameric tandem repeat loci in 4 human population groups" cited in the application see the whole document ---	1,15, 17-24
X	WO,A,92 13969 (BAYLOR COLLEGE MEDICINE) 20 August 1992 see the whole document ---	1,15, 17-24 2-4,16, 25

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FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam

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FIG. 31

1 2 3

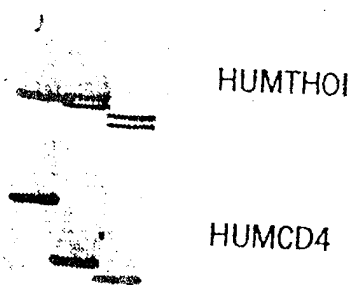
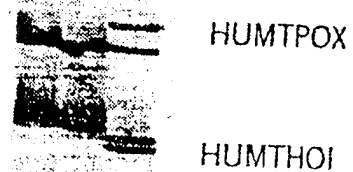


FIG. 32

1 2 3



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FIG. 19

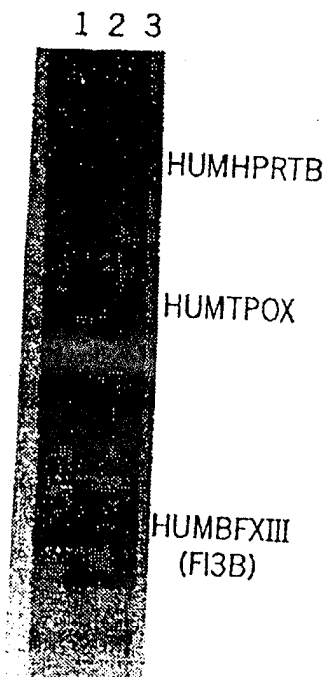


FIG. 20

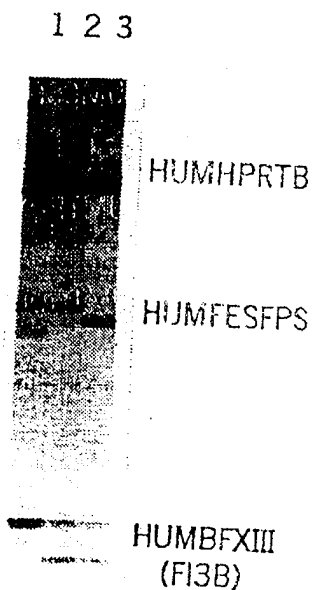


FIG. 21

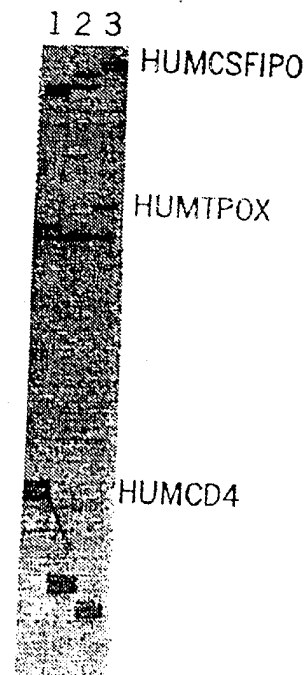


FIG. 22

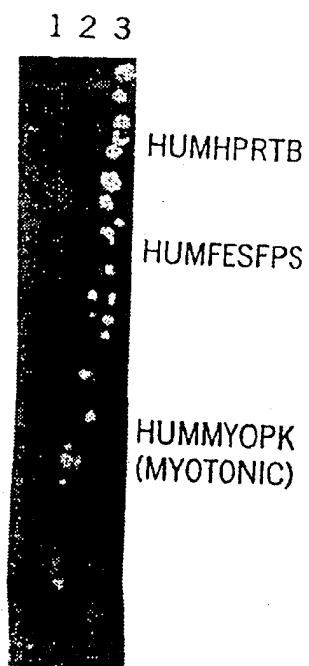


FIG. 23

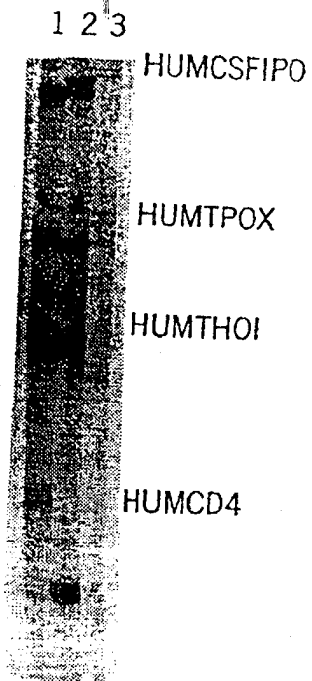
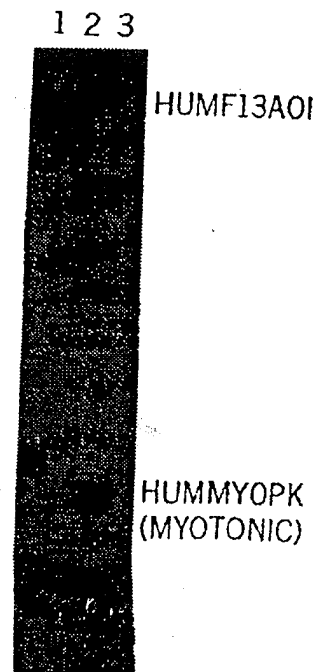


FIG. 24



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FIG. 7

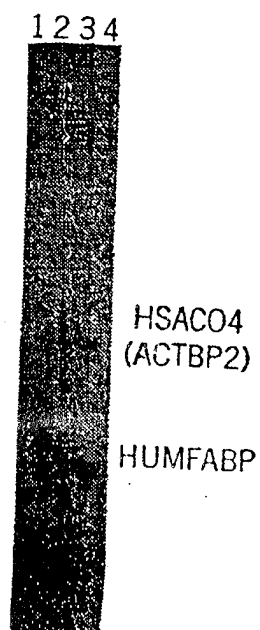


FIG. 8

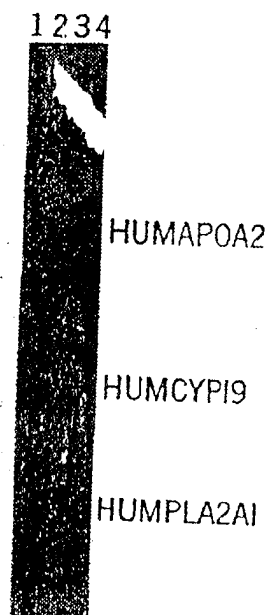


FIG. 9

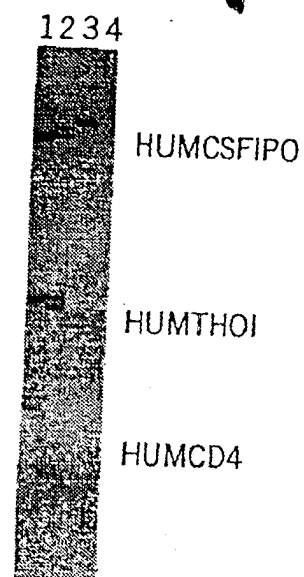


FIG. 10

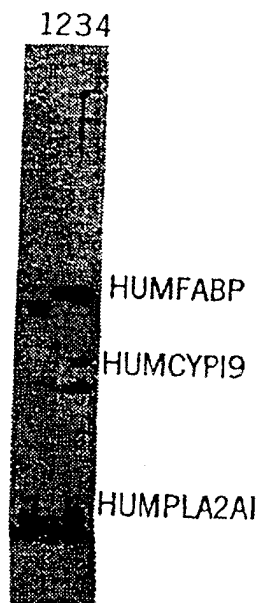


FIG. 11

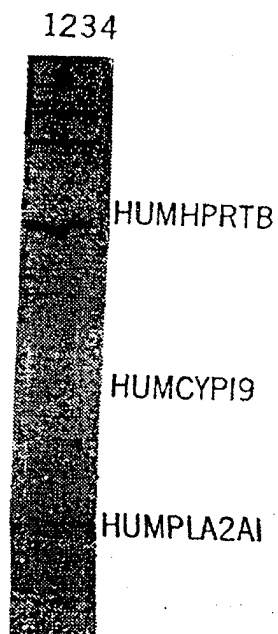
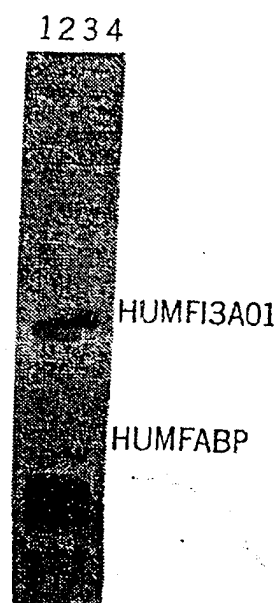
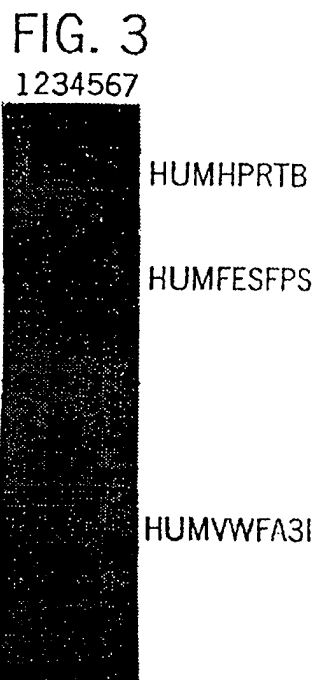
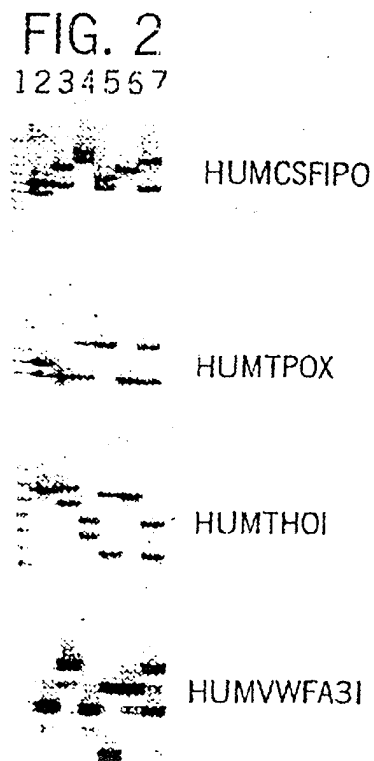
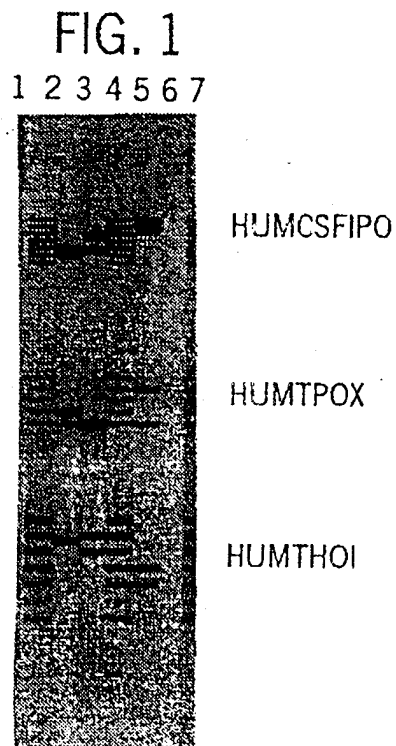


FIG. 12





10 SEQ ID. NO. 11 and SEQ ID. NO. 12, SEQ ID. NO. 13 and
SEQ ID. NO. 14, SEQ ID. NO. 15 and SEQ ID. NO. 16, SEQ
ID. NO. 17 and SEQ ID. NO. 18, SEQ ID. NO. 19 and SEQ
ID. NO. 20, SEQ ID. NO. 21 and SEQ ID. NO. 22, SEQ ID.
NO. 23 and SEQ ID. NO. 24, SEQ ID. NO. 25 and SEQ ID.
NO. 26, SEQ ID. NO. 27 and SEQ ID. NO. 28, SEQ ID. NO.
29 and SEQ ID. NO. 30, and SEQ ID. NO. 31 and SEQ ID.
NO. 32.

- 5 17. The method of claim 1 further comprising adding short
tandem repeat allelic ladders containing nucleotide
fragments of the same lengths as two or more known
alleles for each of the loci and determining the
allele content of the DNA sample by comparison with
the amplified short tandem repeat fragments for each
of the loci.
18. The method of claim 1 wherein the amplified short
tandem repeat sequences are compared by polyacrylamide
gel electrophoresis.
19. The method of claim 1 wherein the amplified short
tandem repeat sequences are compared using silver
stain analysis.
20. The method of claim 1 wherein the amplified short
tandem repeat sequences are compared by fluorescent
analysis.
21. The method of claim 1 further comprising identifying
an appropriate set of loci and primers which provide
non-overlapping alleles.
22. The method of claim 1 wherein the samples to be tested
are selected from the group consisting of blood,
semen, vaginal cells, hair, saliva, urine or other

10 (ACTBP2), HUMCYP19 and HUMPLA2A1; HSAC04 (ACTBP2) and
HUMFABP; HUMAPOA2, HUMCYP19 and HUMPLA2A1; HUMCD4,
HUMCSF1PO and HUMTH01; HUMCYP19, HUMFABP and
HUMPLA2A1; HUMCYP19, HUMHPRTB and HUMPLA2A1;
HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMFESFPS and
15 HUMLIPOL; HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB,
HUMBFXIII (F13B) and HUMPLA2A1; HUMHPRTB, HUMBFXIII
(F13B) and HUMTPOX; HUMHPRTB, HUMBFXIII (F13B) and
HUMFESFPS; HUMCSF1PO, HUMTPOX and HUMCD4; HUMHPRTB,
HUMFESFPS and HUMMYOPK (Myotonic). HUMCSF1PO, HUMTH01
and HUMCD4; HUMCSF1PO, HUMTH01 and HUMVWFA31;
20 HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO,
HUMTPOX, HUMTH01 and HUMVWFA31; HUMHPRTB, HUMFESFPS,
HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX,
HUMTH01 and HUMCD4; and HUMCSF1PO, HUMTH01, HUMTPOX
and HUMCD4.

5. The method of claim 1 wherein the loci are HUMHPRTB and HUMFESFPS.
6. The method of claim 1 wherein the loci are HUMCSF1PO, HUMTPOX, and HUMTH01.
7. The method of claim 6, wherein in said amplifying step, amplification is primed with primer pairs comprising SEQ ID. NO. 5 and SEQ ID. NO. 6, SEQ ID. NO. 29 and SEQ ID. NO. 30, and SEQ ID. NO. 27 and SEQ ID. NO. 28.
8. The method of claim 7, wherein the primer pairs SEQ ID. NO. 5 and SEQ ID. NO. 6, and SEQ ID. NO. 29 and SEQ ID. NO. 30 are present in a concentration of about 0.2 μ M; and the sequence primer pairs SEQ ID. NO. 27 and SEQ ID. NO. 28 are present in a concentration of about 0.6 μ M.

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACTGGCACAG AACAGGCACT TAGG

24

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGAGGAACTG GGAACCACAC AGGT

24

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAAAGCCCTA GTGGATGATA AGAATAATC

29

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGACAGATGA TAAATACATA GGATGGATGG

30

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCTCGAAGGG TCCTTGTAGC CGGG

24

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATAGGTGGG GGTGCGTGGA GGAT

24

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTGACCAAGG ATAGTGGGAT ATAG

24

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGTAACTGAG CGAGACTGTG TCT

23

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGAGGTGGTG TACTACCATA

20

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GATCATGCCA TTGCACTCTA

20

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTAGTATCAG TTTCATAGGG TCACC

25

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGTTCGTTT CCATTGTCTG TCCG

24

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AACCTGAGTC TGCCAAGGAC TAGC

24

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTCCACACAC CACTGGCCAT CTTC

24

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCAGGTACTT AGTTAGCTAC

20

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTACAGTGAG CCAAGGTCGT

20

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Schumm, James W.
Sprecher, Cynthia J.
Lins, Ann M.
- (ii) TITLE OF INVENTION: MULTIPLEX AMPLIFICATION OF
SHORT TANDEM REPEAT LOCI
- (iii) NUMBER OF SEQUENCES: 32
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Ross & Stevens, S.C.
 - (B) STREET: P. O. Box 2599
 - (C) CITY: Madison
 - (D) STATE: Wisconsin
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 53701-2599
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0 Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Sara, Charles S.
 - (B) REGISTRATION NUMBER: 30,492
 - (C) REFERENCE/DOCKET NUMBER: 34506.022
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 608-257-5353
 - (B) TELEFAX: 608-257-9175

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACATCTCCCC TACCGCTATA

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EXAMPLE 30

Fluorescent Detection of Multiplex Amplification
of Loci HUMCSF1PO and HUMTH01

5 In this example, a DNA template was amplified at the individual loci HUMCSF1PO and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in
10 Example 1. Four amplification primers were used in combination, including 2 μ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5] and 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27].

15 Amplified products were detected as in Example 2. Reference is made to Figure 30 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO and HUMTH01.

20

EXAMPLE 31

Fluorescent Detection of Multiplex Amplification
of Loci HUMTH01 and HUMCD4

25 In this example, a DNA template was amplified at the individual loci HUMTH01 and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in
30 Example 1. Four amplification primers were used in combination, including 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 μ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID. 10].

35 Amplified products were detected as in Example 2. Reference is made to Figure 31 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMTH01 and HUMCD4.

ID. 29] and fluorescein-labeled primer 2 [SEQ. ID. 30],
0.5 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-
labeled primer 1 [SEQ. ID. 27] and 0.5 μ M each HUMCD4 primer
1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID.
5 10].

Amplified products were detected as in Example 2.
Reference is made to Figure 26 which is a photograph of a
computer image of a FluorImager scan. Lanes 1-3 contain DNA
samples simultaneously co-amplified for the loci HUMCSF1PO,
10 HUMTPOX, HUMTH01, and HUMCD4.

EXAMPLE 27

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTH01, and HUMCD4

15 In this example, a DNA template was amplified at the
individual loci HUMCSF1PO, HUMTH01, and HUMCD4
simultaneously in a single reaction vessel. The PCR
amplifications were performed as described in Example 1
20 using 0.02U Taq DNA Polymerase/ μ l and amplification
protocol 1, as described in Example 1. Six amplification
primers were used in combination, including 1 μ M each
HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled
primer 1 [SEQ. ID. 5], 1 μ M each HUMTH01 primer 2 [SEQ. ID.
25 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 μ M
each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled
primer 2 [SEQ. ID. 10].

Amplified products were detected as in Example 2.
Reference is made to Figure 27 which is a photograph of a
30 computer image of a FluorImager scan. Lanes 1 and 2
contain DNA samples simultaneously co-amplified for the
loci HUMCSF1PO, HUMTH01, and HUMCD4.

EXAMPLE 28

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTH01, and HUMVWFA31

35 In this example, a DNA template was amplified at the
individual loci HUMCSF1PO, HUMTH01, and HUMVWFA31

each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 1 μ M each HUMMYOPK (Myotonic) primers 1 [SEQ. ID. 21] and 2 [SEQ. ID. 22].

Reference is made to Figure 22 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMMYOPK (Myotonic).

EXAMPLE 23

10 Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4

In this example, a DNA template was amplified at the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 0.04U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in Example 1. Eight primers were used in combination, including 1 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 1 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], 1 μ M each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28], and 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

Reference is made to Figure 23 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4.

EXAMPLE 24

30 Multiplex Amplification of Loci HUMF13A01 and HUMMYOPK (Myotonic)

In this example, a DNA template was amplified at the loci HUMF13A01 and HUMMYOPK (Myotonic) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 0.04U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in Example 1. Four primers were used in combination, including 0.1 μ M each HUMF13A01 primers

using 0.02U Taq DNA Polymerase/ μ l and amplification protocol 2, as described in Example 3. Four primers were used in combination, including 1 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14] and 1 μ M each
5 HUMLIPOL primers 1 [SEQ. ID. 23] and 2 [SEQ. ID. 24].

Reference is made to Figure 18 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B) and HUMLIPOL.

10

EXAMPLE 19

Multiplex Amplification of Loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B)

15 In this example, a DNA template was amplified at the loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example
20 3. Six primers were used in combination, including 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 0.2 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 2 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

25 Reference is made to Figure 19 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B).

30

EXAMPLE 20

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B)

35 In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in

Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

5

EXAMPLE 15

Multiplex Amplification of Loci HUMF13A01, HUMFABP, and HUMCD4

10 In this example, a DNA template was amplified at the loci HUMF13A01, HUMFABP, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 5 using amplification protocol 1, as described in Example 1.
15 Six primers were used in combination, including 1 μ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12], 1 μ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16], and 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

20 Reference is made to Figure 15 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01, HUMFABP, and HUMCD4. Lane 4 displays a sample without DNA subjected to the same
25 procedures, i.e., a negative control.

EXAMPLE 16

Multiplex Amplification of Loci HUMHPRTB and HUMFESFPS

30 In this example, a DNA template was amplified at the loci HUMHPRTB and HUMFESFPS simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1
35 using 500-0.5ng template, 0.02U Taq DNA Polymerase/ μ l and amplification protocol 2, as described in Example 3. Four primers were used in combination, including 0.2 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20] and

displays a sample without DNA subjected to the same procedures, i.e., a negative control.

EXAMPLE 11

5 Multiplex Amplification of Loci HUMCYP19, HUMHPRTB, and HUMPLA2A1

10 In this example, a DNA template was amplified at the loci HUMCYP19, HUMHPRTB, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 9 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], 1 μ M
15 each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], and 1 μ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

20 Reference is made to Figure 11 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCYP19, HUMHPRTB, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

25 EXAMPLE 12

Multiplex Amplification of Loci HUMF13A01 and HUMFABP

30 In this example, a DNA template was amplified at the loci HUMF13A01 and HUMFABP simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 5 using amplification protocol 1, as described in Example 1. Four primers were used in combination, including 1 μ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12], and 1 μ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID.
35 16].

 Reference is made to Figure 12 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified

Four primers were used in combination, 1 μ M each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], and 1 μ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16].

5 Reference is made to Figure 7 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2) and HUMFABP. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a
10 negative control.

EXAMPLE 8

Multiplex Amplification of Loci HUMAPOA2, HUMCYP19, and HUMPLA2A1

15 In this example, a DNA template was amplified at the loci HUMAPOA2, HUMCYP19, and HUMPLA2A simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6
20 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMAPOA2 primers 1 [SEQ. ID. 3] and 2 [SEQ. ID. 4], 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], and 1 μ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2
25 [SEQ. ID. 26].

 Reference is made to Figure 8 which reveals the silver stain detection of the multiplex amplification. Lanes 1 and 3 contain DNA samples simultaneously co-amplified for the loci HUMAPOA2, HUMCYP19, and HUMPLA2A1. Lane 2
30 contains a DNA sample which failed to amplify and lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

EXAMPLE 9

Multiplex Amplification of Loci HUMCD4, HUMCSF1PO, and HUMTH01

35 In this example, a DNA template was amplified at the loci HUMCD4, HUMCSF1PO, and HUMTH01 simultaneously in a

described in Example 2 using amplification protocol 2, as described in Example 3.

Eight primers were used in combination, including 1 μ M each HUMHPRTB primer 2 [SEQ. ID. 20] and fluorescein-labeled primer 1 [SEQ. ID. 19], 2.5 μ M each HUMFESFPS primer 2 [SEQ. ID. 18] and fluorescein-labeled primer 1 [SEQ. ID. 17], 1 μ M each HUMBFXIII (F13B) primer 2 [SEQ. ID. 14] and fluorescein-labeled primer 1 [SEQ. ID. 13], and 0.5 μ M each HUMLIPOL primer 2 [SEQ. ID. 24] and fluorescein-labeled primer 1 [SEQ. ID. 23].

Reference is made to Figure 4 which is a computer image of a FluorImager scan. Lanes 2-7 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL. Lane 1 contains allelic ladders for the 4 loci.

EXAMPLE 5

Multiplex Amplification of Loci HSAC04 (ACTBP2) and HUMCYP19

In this example, a DNA template was amplified at the individual loci HSAC04 and HUMCYP19 simultaneously in a single reaction vessel. The PCR amplifications were performed in 15 μ l volumes with 25ng template, 0.01U Taq DNA Polymerase/ μ l, 1x Taq DNA Polymerase Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100 and 1.5mM MgCl₂) and 200 μ M each of dATP, dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2, as described in Example 3, was employed. Amplified products were separated and detected per example 1. Four primers were used in combination, including 1 μ M each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], and 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8].

Reference is made to Figure 5 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2) and HUMCYP19. Lane 4 displays a

at 60 W and products were visualized by silver stain analysis according the protocol of Bassam et al. (1991).

Reference is made to Figure 1 which reveals the silver stain detection of the multiplex amplification. Lanes 2, 3, and 5 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, and HUMTH01. Lanes 1, 4, and 7 contain allelic ladders for the three loci and lane 6 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

10

EXAMPLE 2

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31

15 In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed in 25 μ l volumes using 25ng template, 0.04U Taq DNA Polymerase/ μ l, 1x STR Buffer (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 20 1.5mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP), and using a Thermal Cyclor 480 (Perkin Elmer Cetus). Amplification protocol 1, as described in Example 1, was employed. Eight amplification primers were used in combination, including 1 μ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 0.15 μ M each HUMTPOX primer 1 [SEQ. ID. 29] and fluorescein-labeled primer 2 [SEQ. ID. 30], 0.2 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27], and 1 μ M each HUMVWFA31 primer 1' [SEQ. ID. 31] and fluorescein-labeled primer 2 [SEQ. ID. 32].

30 Amplified products were separated by denaturing acrylamide gel electrophoresis on a 32cm gel for 45 minutes at 40 watts. Detection of the fluorescent signal was achieved using the FluorImager[™] 575 (Molecular Dynamics, Sunnyvale, CA). Reference is made to Figure 2 which is a computer image of a FluorImager scan. Lanes 2-7 contain DNA samples simultaneously co-amplified for the loci

Kit

The present invention is also directed to kits that utilize the process described. A basic kit includes a container having a locus-specific primer pair (or
5 alternately separate containers containing each primer of a primer pair) for each locus. The kit also includes instructions for use.

Other ingredients may include an allelic ladder directed to each of the specified loci, a sufficient
10 quantity of enzyme for amplification, amplification buffer to facilitate the amplification, loading solution for preparation of the amplified material for gel electrophoresis, human genomic DNA as a control to test that the system is working well, a size marker to insure
15 that materials migrate as anticipated in the gel, and a protocol and manual to educate the user and to limit error in use. The amounts of the various reagents in the kits can be varied depending upon a number of factors, such as the optimum sensitivity of the process. The instructions
20 for use are suitable to enable any analyst to carry out the desired test. It is within the scope of this invention to provide test kits for use in manual applications or test kits for use with automated detectors or analyzers.

25

EXAMPLES

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of
30 the disclosure or protection granted by the patent.

Genomic DNA isolation and quantitation were performed essentially as described by Puers et al., 1993. These methods are generally known to those skilled in the art and are preferred, but not required, for application of the
35 invention.

Amplification products were separated by electrophoresis through a 0.4mm thick 4% denaturing

described in Example 1. Fragment separation occurs based on size and charge of the sample.

The DNA is then detected by, e.g., silver staining (Bassam et al. 1991). Alternatively, if radioactively-labeled or fluorescently-labeled primers were used for each locus, the products are detected by means available to detect these reporters as known to those skilled in the art. Amplified materials may be detected using any of a number of reporters including, e.g., silver staining, radioisotopes, fluorescers, chemiluminescers and enzymes in combination with detectable substrates.

Individual DNA samples containing amplified alleles are preferably compared with a size standard such as a DNA marker or locus-specific allelic ladder to determine the alleles present at each locus within the sample. The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci consists of a combination of allelic ladders for the loci being evaluated.

The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci which are generated using fluorescently-labeled primers for each locus consists of a combination of fluorescently-labeled allelic ladders for the loci being evaluated.

Following the construction of allelic ladders for individual loci, they may be mixed and loaded for gel electrophoresis at the same time as the loading of amplified samples occurs. Each allelic ladder co-migrates with alleles in the sample from the corresponding locus.

A permanent record of the data can be generated with the use of electrophoresis duplicating film (STR systems manual #TMD004, Promega Corporation, Madison, WI.).

Advantage of Fluorescent Detection

With the advent of automated fluorescent imaging, faster detection and analysis of multiplex amplification

multiplex. The derivative multiplexes are, in some sense, routine extensions of the core multiplex.

Preparation of Genomic DNA

5 All methods of DNA preparation which are compatible with the amplification process for a single locus should be appropriate for multiplex amplification. Many examples of preparation methods have been described in the literature (Patel et al. 1984, Gill et al. 1985). DNA concentrations
10 are measured fluorometrically (Brunk et al. 1979).

Amplification of DNA

Human genomic DNA samples are subjected to PCR amplification using primers and thermocycling conditions
15 specific for each locus. Reference is made to Table 1 for details of the primer sequences. The amplification protocol specific to each multiplex is listed in the specific examples.

Successful combinations are generated by trial and error of locus combinations and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified.

5 Of particular importance in the multiplex system is the size range of amplified alleles produced from the individual loci which will be analyzed together. For ease of analysis with current technologies, systems which can be detected by amplification of fragments smaller than 500
10 bases were preferably selected.

The following multiplex combinations have been developed and are considered ideal combinations for use in the present system:

1. HUMTH01 and HUMCSF1PO;
- 15 2. HUMTH01 and HUMCD4;
3. HUMTH01 and HUMTPOX;
4. HUMF13A01 and HUMFABP;
5. HUMF13A01 and HUMMYOPK (Myotonic);
6. HUMF13A01 and HUMBFXIII (F13B);
- 20 7. HUMBFXIII (F13B) and HUMFESFPS;
8. HUMBFXIII (F13B) and HUMLIPOL;
9. HUMHPRTB and HUMFESFPS;
10. HSAC04 (ACTBP2) and HUMCYP19;
11. HSAC04 (ACTBP2) and HUMFABP;
- 25 12. HUMCSF1PO, HUMTPOX and HUMTH01;
13. HUMHPRTB, HUMFESFPS and HUMVWFA31;
14. HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1;
15. HUMAPOA2, HUMCYP19 and HUMPLA2A1;
16. HUMCD4, HUMCSF1PO and HUMTH01;
- 30 17. HUMCYP19, HUMFABP and HUMPLA2A1;
18. HUMCYP19, HUMHPRTB and HUMPLA2A1;
19. HUMF13A01, HUMFABP and HUMCD4;
20. HUMHPRTB, HUMFESFPS and HUMLIPOL;
21. HUMF13A01, HUMFABP and HUMCD4;
- 35 22. HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1;
23. HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX;
24. HUMHPRTB, HUMBFXIII (F13B) and HUMFESFPS;

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are intended to assist in providing a clear and consistent understanding of the scope and detail of the terms:

5 Allelic ladder: a standard size marker consisting of amplified alleles from the locus.

Allele: a genetic variation associated with a segment of DNA, i.e., one of two or more alternate forms of a DNA sequence occupying the same locus.

10 Biochemical nomenclature: standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, deoxyguanosine-5'-triphosphate (dGTP).

15 DNA polymorphism: the condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

Locus (or genetic locus): a specific position on a chromosome. Alleles of a locus are located at identical
20 sites on homologous chromosomes.

Locus-specific primer: a primer that specifically hybridizes with a portion of the stated locus or its complementary strand, at least for one allele of the locus, and does not hybridize efficiently with other DNA sequences
25 under the conditions used in the amplification method.

Polymerase chain reaction (PCR): a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence by $>10^6$ times.
30 The polymerase chain reaction process for amplifying nucleic acid is covered by U. S. Patent Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference for a description of the process.

Polymorphism information content (PIC): a measure of
35 the amount of polymorphism present at a locus (Botstein et al., 1980). PIC values range from 0 to 1.0, with higher values indicating greater degrees of polymorphism. This

migrating next to the corresponding allelic ladder for ease of interpretation in Example 1.

Figure 2 is a computer image showing the fluorescent detection of multiplex amplification of the loci HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31 as detected with a FluorImager™-575 (Molecular Dynamics, Sunnyvale, CA) in Example 2.

Figure 3 is a photograph showing the silver stain detection of the multiplex amplification in Example 3.

Figure 4 is a computer image showing the fluorescent detection of multiplex amplification in Example 4.

Figure 5 is a photograph showing the silver stain detection of the multiplex amplification in Example 5.

Figure 6 is a photograph showing the silver stain detection of the multiplex amplification in Example 6.

Figure 7 is a photograph showing the silver stain detection of the multiplex amplification in Example 7.

Figure 8 is a photograph showing the silver stain detection of the multiplex amplification in Example 8.

Figure 9 is a photograph showing the silver stain detection of the multiplex amplification in Example 9.

Figure 10 is a photograph showing the silver stain detection of the multiplex amplification in Example 10.

Figure 11 is a photograph showing the silver stain detection of the multiplex amplification in Example 11.

Figure 12 is a photograph showing the silver stain detection of the multiplex amplification in Example 12.

Figure 13 is a photograph showing the silver stain detection of the multiplex amplification in Example 13.

Figure 14 is a photograph showing the silver stain detection of the multiplex amplification in Example 14.

Figure 15 is a photograph showing the silver stain detection of the multiplex amplification in Example 15.

Figure 16 is a photograph showing the silver stain detection of the multiplex amplification in Example 16.

Figure 17 is a photograph showing the silver stain detection of the multiplex amplification in Example 17.

It is also an object of the present invention to provide a method and a kit specific for multiplex amplifications comprising specified loci.

These and other objects are addressed by the present invention which is directed to a method of simultaneously analyzing or determining the alleles present at each individual locus of each multiplex. This method comprises the steps of (1) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together; (2) amplifying the STR sequences in the DNA sample; and (3) detecting the amplified materials in a fashion which reveals the polymorphic nature of the systems employed.

The present invention is also directed to a method of simultaneously analyzing multiple STR sequences wherein at least one of the loci is selected from the group consisting of: HUMCSF1PO, HUMTPOX, HUMVWFA31, HUMFESFPS, HUMBFXIII (F13B), HUMLIPOL, HSAC04 (ACTBP2), HUMCYP19, HUMPLA2A1, HUMAPOA2, HUMCD4, HUMF13A01 and HUMMYOPK (Myotonic).

Specifically, the present invention is directed to a method of simultaneously analyzing multiple STR sequences in the following groups of loci: HUMTH01 and HUMCSF1PO; HUMTH01 and HUMCD4; HUMTH01 and HUMTPOX; HUMF13A01 and HUMFABP; HUMF13A01 and HUMMYOPK (Myotonic); HUMF13A01 and HUMBFXIII (F13B); HUMBFXIII (F13B) and HUMFESFPS; HUMBFXIII (F13B) and HUMLIPOL; HUMHPRTB and HUMFESFPS; HSAC04 (ACTBP2) and HUMCYP19; HUMCSF1PO, HUMTPOX and HUMTH01; HUMHPRTB, HUMFESFPS and HUMVWFA31; HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1; HSAC04 (ACTBP2) and HUMFABP; HUMAPOA2, HUMCYP19 and HUMPLA2A1; HUMCD4, HUMCSF1PO and HUMTH01; HUMCYP19, HUMFABP and HUMPLA2A1; HUMCYP19, HUMHPRTB and HUMPLA2A1; HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMFESFPS and HUMLIPOL; HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1; HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX; HUMHPRTB, HUMBFXIII (F13B) and HUMFESFPS; HUMCSF1PO, HUMTPOX and HUMCD4; HUMHPRTB, HUMFESFPS and HUMMYOPK (Myotonic); HUMCSF1PO, HUMTH01 and

Polymorphic STR loci are extremely useful markers for human identification, paternity testing and genetic mapping. STR loci may be amplified via the polymerase chain reaction (PCR) by employing specific primer sequences identified in the regions flanking the tandem repeat.

Alleles of these loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another following electrophoretic separation by any suitable detection method including radioactivity, fluorescence, silver stain, and color.

To minimize labor, materials and analysis time, it is desirable to analyze multiple loci and/or more samples simultaneously. One approach for reaching this goal involves amplification of multiple loci simultaneously in a single reaction. Such "multiplex" amplifications have been described extensively in the literature. Multiplex amplification sets have been extensively developed for analysis of genes related to human genetic diseases such as Duchenne Muscular Dystrophy (Chamberlain et al. 1988, Chamberlain et al. 1989, Beggs et al. 1990, Clemens et al. 1991, Schwartz et al. 1992, Covone et al. 1992), Lesch-Nyhan Syndrome (Gibbs et al. 1990), Cystic Fibrosis (Estivill et al. 1991, Fortina et al. 1992, Ferrie et al. 1992, Morral and Estivill, 1992), and Retinoblasma (Lohmann et al. 1992). Multiplex amplification of polymorphic microsatellite markers (Clemens et al. 1991, Schwartz et al. 1992, Huang et al. 1992) and even STR markers (Edwards et al. 1992, Kimpton et al. 1993, Hammond et al. 1994) have been described.

These amplified products are generally separated by one of several methods of electrophoresis known to those skilled in the art. Several well-known methods of detection of the amplified products have also been described. While ethidium bromide staining of amplified fragments is employed in some cases, in others it is preferred to use methods which label only one of the two strands of the

13. The method of claim 11, wherein the primer pairs SEQ ID. NO. 5 and SEQ ID. NO. 6 are present in a concentration of about $1\mu\text{M}$; primer pairs SEQ ID. NO. 29 and SEQ ID. NO. 30 are present in a concentration of about $0.15\mu\text{M}$, primer pairs SEQ ID. NO. 27 and SEQ ID. NO. 28 are present in a concentration of about $0.2\mu\text{M}$, and primer pair SEQ ID. NO. 31 and SEQ ID. NO. 32 are present in a concentration of about $1\mu\text{M}$.

14. The method of claim 1 wherein the at least two loci include a third locus, and wherein the at least two loci and the third locus are HUMFESFPS, HUMHPRTB, and HUMVWFA31.

15. The method of claim 1 wherein in step (b), the at least two loci are co-amplified by multiplex polymerase chain reaction.

16. The method of claim 10 wherein the at least two loci are co-amplified using one or more oligonucleotide primer pairs selected from the group of primer pairs consisting of:

5 SEQ ID. NO. 5 and SEQ ID. NO. 6,
 SEQ ID. NO. 27 and SEQ ID. NO. 28,
 SEQ ID. NO. 29 and SEQ ID. NO. 30, and
 SEQ ID. NO. 31 and SEQ ID. NO. 32.

17. The method of claim 1 further comprising evaluating short tandem repeat loci using allelic ladders containing nucleotide fragments of the same lengths as two or more known alleles for each of the at least two loci and determining the allele content of each of the at least two loci by comparison with the corresponding short tandem repeat allelic ladder for each of the at least two loci.

25. The kit of claim 24 wherein the oligonucleotide primer pairs are selected from the group of primer pairs consisting of:

- 5 SEQ ID. NO. 1 and SEQ ID. NO. 2,
 SEQ ID. NO. 3 and SEQ ID. NO. 4,
 SEQ ID. NO. 5 and SEQ ID. NO. 6,
 SEQ ID. NO. 7 and SEQ ID. NO. 8,
 SEQ ID. NO. 9 and SEQ ID. NO. 10,
10 SEQ ID. NO. 11 and SEQ ID. NO. 12,
 SEQ ID. NO. 13 and SEQ ID. NO. 14,
 SEQ ID. NO. 15 and SEQ ID. NO. 16,
 SEQ ID. NO. 17 and SEQ ID. NO. 18,
 SEQ ID. NO. 19 and SEQ ID. NO. 20,
15 SEQ ID. NO. 21 and SEQ ID. NO. 22,
 SEQ ID. NO. 23 and SEQ ID. NO. 24,
 SEQ ID. NO. 25 and SEQ ID. NO. 26,
 SEQ ID. NO. 27 and SEQ ID. NO. 28,
 SEQ ID. NO. 29 and SEQ ID. NO. 30, and
 SEQ ID. NO. 31 and SEQ ID. NO. 32.



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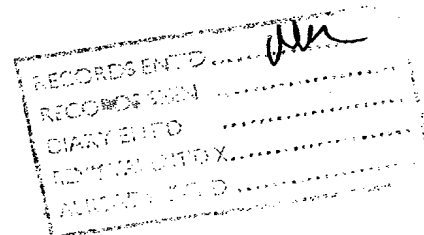
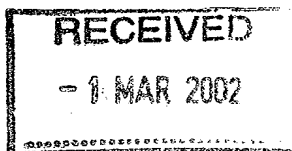
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Datum/Date
26.02.02

Zeichen/Ref./Réf. ME/FP5734553	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°. 97929672.0-2404-US9706293
Anmelder/Applicant/Demandeur/Patentinhaber/Propriétaire/Titulaire PROMEGA CORPORATION	

COMMUNICATION

The European Patent Office herewith transmits as an enclosure the European search report for the above-mentioned European patent application.

If applicable, copies of the documents cited in the European search report are attached.

☒ Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

The following specifications given by the applicant have been approved by the Search Division:

☒ abstract

☒ title

☐ The abstract was modified by the Search Division and the definitive text is attached to this communication.

The following figure will be published together with the abstract: **NONE**

REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.





European Patent
Office

SUPPLEMENTARY

PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent ConventionEP 97 92 9672
shall be considered, for the purposes of subsequent
proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.CI.6)
X	WO 96 10648 A (PROMEGA CORP) 11 April 1996 (1996-04-11) * the whole document *	1-43	C12P19/34 C12Q1/68 C07H21/04
X	KIMPTON P ET AL: "AUTOMATED DNA PROFILING EMPLOYING MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI" PCR METHODS AND APPLICATIONS, COLD SPRING HARBOR, NY, US, vol. 3, no. 1, 1 August 1993 (1993-08-01), pages 13-22, XP000565542 ISSN: 1054-9803 * the whole document *	1-43	
			TECHNICAL FIELDS SEARCHED (Int.CI.6)
			C12Q
The supplementary search report has been based on the last set of claims valid and available at the start of the search.			
INCOMPLETE SEARCH			
The Search Division considers that the present application, or some or all of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for the following claims:			
Claims searched completely :			
Claims searched incompletely :			
Claims not searched :			
Reason for the limitation of the search: see sheet C			
Place of search THE HAGUE		Date of completion of the search 19 February 2002	Examiner Reuter, U
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	



Claim(s) searched incompletely:
1,25

Reason for the limitation of the search:

Present claim 1 relates to an extremely large number of possible sets within a method. Support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC is to be found, however, for only a very small proportion of the methods claimed. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claim which appear to be supported and disclosed, namely those parts relating to the methods for the detection of the sets of loci claimed in claims 2-5.

Present claim 25 relates to products defined by reference to a desirable characteristic or property, namely being suitable for the coamplification of sets of short tandem repeat loci. The claim covers all products having this characteristic or property, whereas the application provides support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC for only a very limited number of such products. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claim also lacks clarity (Article 84 EPC). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claim which appear to be clear, supported and disclosed, namely those parts relating to the products that are the sets of primers used in the examples 1-35 and claimed in claim 26.

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 97 92 9672

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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19-02-2002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2118048 A1	31-03-1996
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